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<b>(21) International Application Number:</b> PCT/FI99/00204 <b>(22) International Filing Date:</b> 17 March 1999 (17.03.99) <b>(30) Priority Data:</b> 980604 18 March 1998 (18.03.98) FI <b>(71) Applicant (for all designated States except US):</b> HELSINKI UNIVERSITY LICENSING LTD. OY [FI/FI]; Koetilantie 3, FIN-00710 Helsinki (FI). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KOIVUNEN, Erkki [FI/FI]; Ohrakatu 8 E 52, FIN-05880 Hyvinkää (FI). SORSA, Timo [FI/FI]; Lounaisväylä 17, FIN-00200 Helsinki (FI). SALO, Tuula [FI/FI]; Fyysikontie 8, FIN-90570 Oulu (FI). <b>(74) Agent:</b> OY JALO ANT-WUORINEN AB; Iso Roobertinkatu 4-6 A, FIN-00120 Helsinki (FI).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> NOVEL MATRIX METALLOPROTEINASE INHIBITORS AND DOWN-REGULATORS		
<b>(57) Abstract</b> <p>The present invention relates to novel matrix metalloproteinase (MMP) inhibitors and down-regulators, to a process for the preparation of these inhibitors, to pharmaceutical compositions comprising these inhibitors/down-regulators, to the use of the novel MMP inhibitors for the manufacture of pharmaceutical and research preparations, to a method for inhibiting and down-regulating MMP-dependent conditions either <i>in vivo</i> or <i>in vitro</i>, to a method for inhibiting formation, synthesis, expression activations, and/or functions as well actions of matrix metalloproteinases, and to the use of the novel MMP inhibitors and down-regulators in biochemical isolation and purification procedures of matrix metalloproteinases.</p>		

## Novel matrix metalloproteinase inhibitors and down-regulators

The present invention relates to novel matrix metallopro-  
teinase (MMP) inhibitors and down-regulators, to a pro-  
cess for the preparation of these inhibitors, to pharma-  
ceutical compositions comprising these inhibitors/down-  
regulators, to the use of the novel matrix metallopro-  
teinase inhibitors for the manufacture of pharmaceutical  
and research preparations, to a method for inhibiting and  
down-regulating MMP-dependent conditions either *in vivo*  
or *in vitro*, to a method for inhibiting formation, synt-  
hesis, expression and/or functions as well as actions of  
matrix metalloproteinases, and to the use of the novel  
MMP inhibitors in biochemical isolation and purification  
procedures of matrix metalloproteinases.

Matrix metalloproteinases (MMPs) constitute a superfamily  
of genetically closely related proteolytic enzymes capa-  
ble of degrading almost all the constituents of extracel-  
lular matrix and basement membrane that restrict cell  
movement. MMPs also process serpins, cytokines and growth  
factors as well as certain cell surface components  
(Woessner, 1991; Birkendal-Hansen, 1995; Chandler et al.,  
1997). MMPs are thought to have a key role in mediating  
tissue remodeling and cell migration during morphogenesis  
and physiological situations such as wound healing, trop-  
hoblast implantation and endometrial menstrual breakdown.

MMPs are further involved in processing and modification  
of molecular phenomena such as tissue remodeling, angio-  
genesis, cytokine, growth factor, integrin and their re-  
ceptor processing (Chandler et al., 1997). MMPs also me-  
diate release and membrane-bound proteolytic processing  
of tumor necrosis factor (TNF- $\alpha$ ) by bacterial-virulence  
factor induced monocytes. This event is mediated by a  
membrane-bound metalloproteinase TACE (TNF- $\alpha$  activating

enzyme). Thus MMP-inhibitors, such as the novel peptides presented in this invention, can i.a. prevent activation of TNF- $\alpha$  by blocking this type of activating enzymes (Shapira et al., 1997).

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Several studies have shown that the expression and activities of MMPs are pathologically elevated over the body's endogenous anti-proteinase shield in a variety of diseases such as cancer, metastasis, rheumatoid arthritis, multiple sclerosis, periodontitis, osteoporosis, 10 osteosarcoma, osteomyelitis, bronchiectasis, chronic pulmonary obstructive disease, and skin and eye diseases. Proteolytic enzymes, especially MMPs, are believed to contribute to the tissue destruction damage associated 15 with these diseases.

There is a variety of other disorders in which extracellular protein degradation/destruction plays a prominent role. Examples of such diseases include arthritides, acquired immune deficiency syndrome (AIDS), burns, wounds 20 such as bed sores and varicose ulcers, fractures, trauma, gastric ulceration, skin diseases such as acne and psoriasis, lichenoid lesions, epidermolysis bollosa, aftae (reactive oral ulcer), dental diseases such as periodontal diseases, peri-implantitis, jaw and other cysts and 25 root canal treatment or endodontic treatment, related diseases, external and intrinsic root resorption, caries etc.

30 At least 20 members of the MMP-superfamily are known (Birkendal-Hansen, 1995; Pei & Weiss, 1996; Llano et al., 1997), and the number of MMP-family members and their cellular origins is growing all the time. Each of the MMP enzymes contains a putative tridentate Zn<sup>2+</sup> binding site 35 which is believed to constitute the active site in the enzyme. Very recently, three new members of the MMP-family were discovered by screening cDNA libraries for homo-

logies to conserved regions of the known MMP genes and named the membrane-type matrix metalloproteinases-1, -2, and -3 (MT-MMP-1, -2, and-3). Based on their predicted amino acid sequences, each of the MT-MMPs, like almost all previously characterized MMPs, contains (i) a candidate leader sequence, (ii) a propeptide region which includes a highly conserved PRCGXPD sequence that helps to stabilize the MMP zymogen in a catalytically inactive state, (iii) a zinc-binding catalytic domain, and (iv) a hemopexin-like domain near their respective C-termini. In addition, in a pattern similar to that described for stromelysin-3, each of the MT-MMPs contains a short amino acid insert sandwiched between their pro- and catalytic domains that encodes a potential recognition motif for members of the proprotein convertase family. Despite their considerable similarity to other MMP family members, however, only the MT-MMPs contain approximately 75-100 amino acid extensions at their C-termini, each of which includes a hydrophobic stretch consistent with the presence of a transmembrane (TM) domain. Thus, in contradistinction to all other MMPs, the MT-MMPs are expressed as membrane-associated ectoenzymes rather than soluble proteins (Pei & Weiss, 1996).

A comprehensive review of the MMP-family members, their activation, modes of action, their inhibition by various natural proteins (endogenous inhibitors) and synthetic compounds as well as details of the involvement of MMP family members in various pathological conditions and diseases is given by Woessner (1991); Krane (1994); Birkendal-Hansen *et al.* (1993); and Birkendal-Hansen (1995), the whole disclosures of which are incorporated herein by reference. In the scope of the present invention the term matrix metalloproteinase (MMP) refers to all discovered MMPs.

The gelatinase A or 72 kDa MMP-2 and gelatinase B or 92 kDa MMP-9 were originally described as type IV collagenases because they appeared to be essential enzymes for the degradation of the basement membrane (Tryggvason et al., 1987). Cells need to traverse the endothelial basement membrane during entry to and exit from the circulation. This is also a critical key step in the metastatic cascade tumor cells have to accomplish before they can metastasize to distant organs. MMP-2 and MMP-9 may also have a function in other steps of the metastatic cascade such as in angiogenesis (Hanahan & Folkman, 1996; Volpert et al., 1996) and local tumor invasion (Stetler-Stevenson et al., 1993).

Because MMPs are potential targets for therapeutic intervention, much work has been focused on the design of synthetic metalloproteinase inhibitors. Many MMP-inhibiting compounds containing reactive zinc-chelating groups such as thiol, hydroxamate, EDTA, phosphoramidate, phosphinate etc. have been developed (Beckett et al., 1996). Some of the peptidomimetics have shown beneficial effects in animal models of metastasis, arthritis, and other inflammatory diseases. Tumor cell invasion can also be inhibited by the native MMP inhibitors TIMP-1 (tissue inhibitor of metalloproteinase) and TIMP-2. MMPs can also be inhibited by peptides based on the highly conserved pro-domain region of MMPs that is important for latency of MMPs (Park et al., 1991; Melchiori et al., 1992; Fotouhi et al., 1994). In addition, tetracyclines and their non-antimicrobial chemically-modified (CMT) as well as anthracycline derivatives have been found to inhibit MMPs (Golub et al., 1992; Sorsa et al., 1994).

Although the above discussion shows that some inhibitors for MMPs do exist and have been investigated, the tests are still mostly at the experimentation stage and no clinically acceptable inhibitor for MMPs exists as a thera-

peutic or prophylactic drug for any of the pathological states and diseases potentially connected with MMPs. Adverse side effects which have been detected in the above described MMP inhibitors include, for instance, toxicities (synthetic peptides), antimicrobial activities (tetracyclines), etc.

An alternative to rational molecular design is to screen libraries of random peptides or other chemicals to find lead compounds binding to target molecules. In particular, peptide libraries displayed on the surface of bacteriophage have often yielded valuable binding peptides to target proteins. However, it has been more difficult to isolate inhibitors to proteinases from libraries of short peptides, possibly because short peptides are easily degraded by proteinases. Phage-displayed peptide libraries have rather been utilized to obtain information of the sequences cleaved by a proteinase (Matthews & Wells, 1993; Smith et al., 1995). Inhibitors to proteinases have been developed with phage surface expression and selection of large proteinase inhibitor domains in which certain active site residues have been randomized (Roberts et al., 1992; Dennis et al., 1995).

The present inventors have now successfully isolated novel peptide inhibitors to MMPs, especially to MMP-9 and MMP-2, using phage-displayed libraries of peptides that were conformationally restrained by designed disulfide bonds. The most active MMP inhibitors developed are capable of inhibiting *in vitro* migration of endothelial cells as well as invasion of tumor cells, thus being potential lead compounds to design peptidomimetics to block MMPs. The peptides can also be used in column chromatographic matrices for biochemical isolation and purification procedures of MMPs.

It is therefore an object of the present invention to provide novel matrix metalloproteinase inhibitors and binding-ligands based on the cyclic structure (disulfide bond between cysteines) of the peptide motif

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CXXHWGFXXC

which corresponds to the sequence shown in SEQ ID No. 1 of the sequence listing and wherein X is any amino acid residue.

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It is another object of the present invention to provide novel matrix metalloproteinase inhibitors and down-regulators based on the cyclic structure of the peptide motifs

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CRRHWGFEFC

which corresponds to the sequence shown in SEQ ID No. 2, and

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CTTHWGFTLC

which corresponds to the sequence shown in SEQ ID No. 3.

The present invention also relates to a pharmaceutical composition comprising an amount of the novel matrix metalloproteinase inhibitor(s)/down-regulator(s) effective to reduce the activities, activations, functions, and/or expressions of one or more MMPs, especially of MMP-2 and/or MMP-9, and a pharmaceutically and biochemically acceptable carrier. Pharmaceutical compositions comprising novel MMP inhibitor(s)/downregulator(s) according to the invention may be used systemically, locally and/or topically. They also include all potential combinations (combo-medications) with other MMP-inhibitors, other drugs and tumor-homing chemicals/molecules.

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- The present invention also includes the use of the novel matrix metalloproteinase inhibitors for the manufacture of pharmaceutical preparations for the treatment of matrix metalloproteinase dependent conditions, and also
- 5 their use, for example as affinity ligands, in biochemical purification and isolation procedures of MMPs. The MMP-dependent conditions include, but are not limited to, wounds, burns, fractures, lesions, ulcers, cancer and metastasis progression in connective tissues and bone,
- 10 periodontitis, gingivitis, peri-implantitis, cysts, root canal treatment, internal and external root canal resorption, caries, AIDS, corneal ulceration, gastric ulceration, aftae, trauma, acne, psoriasis, loosening of the endosseal hip-prosthesis, osteomyelitis, osteoporosis, tissue remodeling, angiogenesis, arthritides (rheumatoid,
- 15 reactive and osteo arthritides), angiogenesis, lung diseases (bronchiectasis and chronic obstructive pulmonary diseases and other lung diseases).
- 20 The present invention also relates to a process for the preparation of novel matrix metalloproteinases which process comprises standard solid-phase Merrifield peptide synthesis.
- 25 The novel CXXHWGFXXC structure according to the invention does not show similarity to previously described MMP inhibitors, although the activities of CTTHWGFTLC resemble the properties of chemically modified tetracyclines (CMTs) as will be described below. The peptides comp-
- 30 rising the novel structure were derived from the single cysteine-expressing CX<sub>2</sub> library and exhibited a HWGF consensus sequence. All contained a second cysteine showing a cyclic structure CXXHWGFXXC. Phage attachment experiments indicated that the cloned phages bound to MMP-9
- 35 with considerable affinity.



The cyclic peptides according to the invention inhibited degradation of gelatin and casein substrates by MMP-2 and MMP-9 with  $IC_{50}$  of 5-10  $\mu$ g/ml. Of a series of peptides synthesized, the HWGF-containing peptides CRRHWGFEFC and CTTHWGFTLC were found to be most promising as inhibitors of MMP-9. These two HWGF-containing peptides also inhibited MMP-2. The fact that the peptides were selected on MMP-9 but can strongly inhibit also MMP-2 indicates that the peptides recognize a binding site very similar between MMP-9 and MMP-2.

The most active HWGF-containing peptide developed (CTTHWGFTLC) inhibited cell migration studied in normal serum-containing media, and blocked the migration of human endothelial cells as well as invasion of HT1080 fibrosarcoma and C8161 melanoma cells through a reconstituted basement membrane. These findings imply that both cancer cells and endothelial cells may use quite a similar MMP-dependent mechanism to migrate that is sensitive to the down-regulating effect of CTTHWGFTLC. The high activity of CTTHWGFTLC could at least partially be due to the fact that the peptide can not only inhibit an active enzyme but can interfere with the autoactivation of purified proMMP-9 and proMMP-2 as is shown below by using gelatin and casein substrates. The peptide can also down-regulate the production of MMP-9. In contrast to the phage binding data in which we were unable to see any phage binding to proMMP-9, the synthetic CTTHWGFTLC peptide does bind to proMMP-9 as indicated by single-step isolation of proMMP-9 from human leukocyte buffy coats using affinity chromatography with the peptide coupled to Sepharose. On the whole, it is possible that by binding to proMMPs the peptide can hinder the true proteolytic activation by other proteinases that is the likely activation mechanism during cell invasion.

The corresponding linear peptides were virtually inactive as demonstrated by a loss of activity after reduction and alkylation of the cysteines. Especially preferred MMP inhibitors according to the present invention are thus the cyclic peptide inhibitors CTTHWGFTLC and CRRHWGFEFC, which inhibit the activity of MMP-2 and MMP-9 as shown below.

As stated above, the novel cyclic peptide inhibitors we have developed are useful lead compounds to design peptidomimetics to block MMPs and cell migration. The CXXHWGFXXC motif may also be utilized to develop more selective inhibitors to individual members of the MMP family, as MMP-2 and MMP-9 were differently inhibited by the two CXXHWGFXXC peptides: MMP-2 was more strongly inhibited by CTTHWGFTLC while MMP-9 was preferentially inhibited by CRRHWGFEFC. Selective inhibitors directed e.g. to MMP-2 might be more efficient in preventing tumor dissemination, as in many experimental systems the metastatic potential of tumor cells rather correlated with MMP-2 activity rather than with MMP-9 activity. Finally the small size of the MMP-targeting cyclic peptides can be utilized to carry drugs to tumors. Phage-library derived peptides targeting receptors in tumor vasculature have been found to be useful cytotoxic drug carriers to tumors in mice. MMPs are potential receptors for targeted chemotherapy, because they are usually overexpressed in tumors as compared to normal tissues and appear to be involved in the angiogenic process.

Thus, as a result of the invention, MMP dependent conditions may now be treated or prevented either with the novel MMP inhibitors alone or in combination with other drugs normally used in connection with the disease or disorder in question. These include for example tetracyclines, chemically modified tetracyclines (Golub et al., 1992), bisphosphonates, as well as homing/carrier molecu-

les to the sites of tumors, such as integrin-binding peptides (Arap et al., 1998). The amount of novel matrix metalloproteinase inhibitors to be used in the pharmaceutical compositions according to the present invention varies depending on the specific inhibitor used, the patient and disease to be treated as well as the route of administration.

The novel MMP inhibitors of the present invention have shown no toxicity when injected into animals and do not affect cell number or viability as determined by trypan blue dye exclusion.

The present invention thus also relates to a method for the therapeutic or prophylactic treatment of MMP-dependent conditions in mammals by administering to said mammal an effective amount of the novel MMP-inhibitor(s), as well as to a method for inhibiting the formations, synthesis, expressions, activations, functions and actions of MMPs in mammals by administering the novel MMP-inhibitor(s)/down-regulator(s) in an amount which is effective in blocking the formation, activation and actions of MMPs.

The present invention also relates to a method for inhibiting matrix metalloproteinases *in vitro* comprising adding to an *in vitro* system the novel matrix metalloproteinase inhibitor(s) in an amount which is effective in inhibiting the MMP activity.

A further object of the invention is a method for isolating and purifying matrix metalloproteinases with the aid of the novel matrix metalloproteinase inhibitor(s).

### Brief description of the figures

Fig. 1 shows the results from the inhibition of MMP-9-mediated [ $^{125}$ I]-gelatin degradation using synthetic peptides. APMA-activated MMP-9 was preincubated with the CRRHWGFEFC and CTTHWGFTLC at the concentrations indicated for 1 h before adding [ $^{125}$ I]-gelating substrate. After gelatinolysis for 1 h, the counts released into medium were determined. The results show means from duplicate measurements. Similar results were obtained in three independent experiments.

Fig. 2 shows gelatinolysis induced by APMA-activated MMPs or their proforms. The concentrations of the cyclic and linear CRRHWGFEFC peptide were 10  $\mu$ g/ml. The results show means from duplicate experiments.

Fig. 3 shows inhibition of MMP-2-mediated casein degradation by CTTHWGFTLC (A, B) and CRRHWGFEFC (C, D). After 1 h pretreatment with the peptides, APMA-activated MMP-2 (A, C) or proMMP-2 (B, D) was incubated with the casein for 2 h. 52  $\mu$ M  $\beta$ -casein was used as substrate for MMPs. Shown is Coomassie Blue-staining of the 21 kD  $\beta$ -casein (lane 1) and its fragments (lanes 2-9) resolved by SDS-PAGE (A, B); CTTHWGFTLC was used at the concentrations of (2) 0  $\mu$ g/ml, (3) 75, (4) 50, (5) 25, (6) 10, (7) 5, (8) 1, and (9) 0.5  $\mu$ g/ml in the lanes 2-9, respectively. (C, D); the concentrations of CRRHWGFEFC were 0, 250, 100, 50, 25, 10, 1, and 0.5  $\mu$ g/ml, respectively.

Fig. 4 shows binding of proMMP-9 to CTTHWGFTLC peptide coupled to Sepharose. Lysate of human buffy coat cells was applied to each peptide Sepharose, and the bound proteins were analyzed on SDS gels followed by Coomassie Blue staining (lanes 1-2), or immunoblotting with anti-MMP-9 antibodies (lanes 5-6). Lanes 1 and 5 show proteins eluted from CTTHWGFTLC-Sepharose. Lanes 2 and 6 show pro-

teins eluted from GACLRSGRGCGA-Sepharose. Lane 3 shows protein staining of the cell lysate. Lane 4 displays the molecular weight markers of 200, 92, 76, and 55 kDa.

5 Fig. 5 shows how CTTHWGFTLC inhibits migration of HT1080  
fibrosarcoma cells. Cells were pretreated with CTTHWGFTLC  
at the concentrations indicated or with 500  $\mu$ g/ml of the  
unrelated EVGTGSCNLECVSTNPLSGTEQ control peptide for 2 h.  
Cells were plated on transwell chambers and allowed to  
10 migrate for 20 h in 10 % serum-containing medium. Cells  
that traversed to the undersurface of the filter were  
stained and the filter area was scanned. The results show  
mean optical density  $\pm$  S.D. from triplicate wells. The  
optical density of blank Transwell without cells was of  
15 0.000.

Fig. 6 shows comparison of the efficacy of the MMP inhi-  
bitors CTTHWGFTLC and CMT-8 to prevent migration of C8161  
melanoma cells. Cells were pretreated with CTTHWGFTLC,  
20 CMT-8, or with the EVGTGSCNLECVSTNPLSGTEQ control, and  
allowed to migrate 20 h in Transwell chambers. Cells that  
migrated to the undersurface of the filter were stained  
and scanned. The results show mean optical density  $\pm$  S.D.  
from triplicate wells.

25 Fig. 7 shows inhibition of endothelial cell migration by  
CTTHWGFTLC. Endothelial cells were allowed to migrate for  
18 h in the presence of 20 % serum for HUVEC, or 10 % se-  
rum for EaHy92 line. Shown is the relative number of  
30 cells having traversed to the undersurface of Transwell  
chambers. The results show means  $\pm$  SD from triplicate  
wells.

Fig. 8 shows inhibition of Matrigel invasion of tumor  
35 cells by CTTHWGFTLC. C8161 or HT1080 cells were allowed  
to invade through Matrigel for 24 h in 10 % serum-con-  
taining medium. The concentrations of CTTHWGFTLC and the

EVGTGSCNLECVSTNPLSGTEQ control were 500  $\mu$ g/ml. The invaded cells were counted, and the relative number of cells are expressed as means  $\pm$  S.D. from triplicate wells.

- 5 Fig. 9 shows that breast carcinoma growth is clearly inhibited by CTTHWGFTLC peptide.

- 10 Fig. 10 shows gelatin zymography of melanoma cell conditioned medium. CTTHWGFTLC peptide but not the control peptide inhibits the formation of active 82 kD MMP-9.

- 15 Figs 11A, 11B and 11C show MB-435 breast carcinoma cells grown for 2 days in 10 % serum in the absence or presence of CTTHWGFTLC peptide.

- Fig. 12 shows the effect of CTTHWGFTLC (P291) on keratinocyte gelatinase production and expression.

- 20 Fig. 13 shows the effect of CTTHWGFTLC (P291) on keratinocyte migration. The photograph of the plates is taken after 4 days of migration.

- The following examples illustrate the invention without, however, limiting it in any way.

- 25 **Example 1.** Preparation of phage display libraries and selection of MMP-9-binding phage

- 30 The single cysteine-expressing CX<sub>2</sub> library was prepared according to the methods described previously (Koivunen et al., 1994a; Koivunen et al., 1994b; and Koivunen et al., 1995, which are all incorporated herein by reference).

- 35 ProMMP-9 was purified from human neutrophils and activated with aminophenyl mercuric acetate (APMA) essentially as described by Hibbs et al. (1985). For the selection of

MMP-9 binding phage, APMA-activated MMP-9 was coated on microtiter wells overnight at 4°C using a concentration of 1 µg/ml, after which the cells were saturated with 5 % bovine serum albumin. In the first panning, the library  
5 was incubated overnight at 4 °C in 50 mM Tris-HCl / 0.1 M NaCl buffer (pH 7.5) (TBS) containing 1 % bovine serum albumin, and after extensive washing the bound phage were eluted with low pH buffer. In the subsequent pannings, the amplified phage were allowed to bind for 1 h at 22  
10 °C. Randomly selected clones were amplified overnight and sequenced as described by Koivunen et al. (1994b). The binding of each clone to the MMP-9 was verified by attachment assay, in which the cloned phage were incubated for 60 min in MMP-coated or in blank microtiter wells.  
15 The wells were washed five times with TBS containing 0.5 % Tween 20. The bound phage were quantitated by adding 50 ng per well of anti-M13 antibody (Pharmacia, Uppsala, Sweden) labeled with an Europium-chelate (Wallac Ltd., Turku, Finland). After incubation for 45 min followed by  
20 washing, the fluorescence was measured with 1230 Arcus fluorometer (Wallac Ltd., Turku, Finland).

Three MMP-9 binding sequences, CRRHWGFEFC, CTTHWGFTLC and CSLHWGFWWC, were derived from the CX<sub>3</sub> library. All three  
25 contained a second cysteine showing a cyclic structure CXXHWGFXXC. In spite of several attempts, we could isolate only three HWGF-containing phage apparently because of the dominance of the LRSGRG motif in the selected clones. We therefore constructed a peptide library, where random  
30 tetrapeptides (and thus also HWGF) were flanked on both sides by cysteine residues, which could make several disulfide bridges and thereby constrict the peptide conformation. This CX<sub>3</sub>CX<sub>4</sub>CX<sub>2</sub>C library expressed three different peptide ring sizes with two, three and four random resi-  
35 dues. On panning with MMP-9, this library yielded the WGF, YGF and FGF motifs, which are similar to the HWGF consensus except that histidine was not conserved.

**Example 2.** Synthesization of peptides and determination of their MMP inhibitor activity by enzyme inhibition assays

5 We synthesized cyclic peptides corresponding to those phage motifs of Example 1 that showed the highest avidity for MMP-9, and determined the metalloproteinase inhibitor activity of the synthetic peptides using gelatin and casein degradation assays.

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Peptides were synthesized on an Applied Biosystems model 433A (Foster City, CA) using Fmoc-chemistry and cyclized in 5 % acetic acid (pH 6.0) containing 20 % dimethyl sulfoxide overnight at room temperature with constant mixing. After dilution 1:2 with 0.1 % trifluoro acetic acid, peptides were purified with reverse-phase HPLC. The structures of the peptides were confirmed by mass spectrometry. Peptides were stored in a stock solution of 100 mg/ml in H<sub>2</sub>O, and were diluted to buffers with neutral pH just before use.

20

For the gelatin and casein degradation assays, purified MMP-2 and MMP-9 (50-100 ng) were first incubated for 60 min with various concentrations of the peptide inhibitors, after which a 21 kDa  $\beta$ -casein (52  $\mu$ M) or [<sup>125</sup>I]-gelatin substrate was added. After incubation for 2 h at 22 °C, degradation of the casein was analyzed by SDS gel electrophoresis. The degradation of [<sup>125</sup>I]-gelatin was determined by counting radioactivity in the supernatant after precipitation of undegraded gelatin with 20 % trichloroacetic acid.

30

Of a series of peptides synthesized, the HWGF motif-containing peptides CRRHWGFEFC and CTHWGFTLC were found to be most promising inhibitors of MMP-9. In the [<sup>125</sup>I]-gelatin degradation assay, CRRHWGFEFC was the more active of the two peptides and inhibited APMA-activated MMP-9 with

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a half-maximal inhibitory value ( $IC_{50}$ ) of about 10  $\mu$ g/ml (Fig. 1).

5 The HWGF-containing peptides also inhibited the gelatinolytic activity mediated by proMMP-9 that autoactivates during incubation with gelatin. Fig. 2 shows that more than 50 % inhibition of proMMP-9 activity was obtained with the CRRHWGFEFC peptide at a concentration of 10  $\mu$ g/ml. To assess the importance of the disulfide bond for the activity of the CRRHWGFEFC peptide, we prepared a linearized version of the peptide by reducing and alkylating the cysteine residues as described by Koivunen et al. (1993). Linearization of the peptide resulted in a loss of inhibitory activity against proMMP-9 as well as the APMA-activated enzyme (Fig. 2).

20 proMMP-2 was purified from serum-free culture medium of human gingival fibroblasts. The two HWGF-containing peptides CRRHWGFEFC and CTTHWGFTLC also inhibited MMP-2, and at a concentration of 10  $\mu$ g/ml the cyclic CRRHWGFEFC peptide blocked gelatinolysis by both proMMP-2 and APMA-activated MMP-2 (Fig. 2). The linear peptide used as a control was virtually inactive.

25 We also used  $\beta$ -casein as a substrate for the gelatinases and analyzed the degradation products by SDS gel electrophoresis. The two HWGF-containing peptides effectively prevented the degradation of casein by the MMPs. For MMP-2, the CTTHWGFTLC and CRRHWGFEFC peptides had  $IC_{50}$  values of about 5  $\mu$ g/ml and 25  $\mu$ g/ml, respectively (Figs. 3A and 3C). proMMP-2 not preactivated with APMA also caused casein degradation, and this was blocked by the peptides at the same  $IC_{50}$  of 5  $\mu$ g/ml and 25  $\mu$ g/ml, respectively (Figs. 3B and 3D). Caseinolysis by MMP-9 was similarly inhibited by the peptides at low micromolar concentrations except that CRRHWGFEFC was a slightly more potent inhibitor for this MMP than CTTHWGFTLC. These peptides (0-200  $\mu$ g/ml)

did not inhibit membrane-type matrix metalloproteinase-1 (MT1-MMP), providing evidence for the importance of gelatinases (MMP-9 and -2) in tumor invasion and basement membrane destruction.

5

**Example 3. Extraction of proMMP-9 by peptide affinity chromatography**

To demonstrate that the synthetic peptides selected from the phage libraries recognize MMP-9, we performed affinity chromatography with the peptides coupled to Sepharose.

Affinity chromatography resin of CTTHWGFTLC was prepared by coupling 2 mg peptide per 1 ml of CNBr-activated Sepharose according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). Human buffy coat cells obtained from Finnish Red Cross were lysed in 50 mM TBS containing 1 % octyl glucoside, and 20 ml of the cleared extract was applied to each peptide Sepharose. The columns were washed until the OD<sub>280</sub> was below 0.01. The bound proteins were eluted with 0.1 M glycine-HCl buffer, pH 2.2, in the presence of 1 % octyl glucoside. The pH was then neutralized with 1 M Tris base. Twenty  $\mu$ l of the fractions were analyzed by SDS gel electrophoresis on 8 % acrylamide gels under reducing conditions. Proteins were stained with Coomassie Blue. For immunoblot analysis, nitrocellulose filters were incubated with polyclonal MMP-9 antibodies at a 1:500 dilution for 1 h followed by secondary antirabbit antibodies at a 1:1000 dilution for another 1 h. The enhanced chemiluminescence system (Amersham, Buckinghamshire, England) was used for visualization.

Extracts from leukocytes were applied to Sepharose columns coupled with CTTHWGFTLC and the proteins bound were analyzed by SDS gel electrophoresis and immunoblotting with anti-MMP-9 antibodies. The peptide column bound a

set of polypeptides one of which was the 92 kDa proMMP-9 (Fig. 4). ProMMP-9 bound to the CTTHWGFTLC Sepharose migrated on SDS gels as a doublet at 92 kDa (lane 1), both forms of which were immunoreactive with anti-MMP-9 antibodies (lane 6). A similar doublet can be observed in MMP-9 immunoblots of culture medium conditioned by several tumor cell lines (data not shown). The peptide Sepharose also bound a set of polypeptides migrating at 55-65 kDa, the identity of which are not known and were not studied further.

#### Example 4. Inhibition of cell migration by CTTHWGFTLC

To test the effectiveness of the novel HWGF-containing MMP inhibitors on cellular migration, we chose to use the CTTHWGFTLC peptide because of its better solubility.

The endothelial cell line HUVEC (human umbilical vein endothelial cells, obtained from the ATCC, Rockville, MD) was grown in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (100 µg/ml), 10 mM HEPES, 30 µg/ml endothelial cell growth supplement (Biomedical Technologies, Stoughton, MA), and 20 % fetal calf serum. The HT1080 fibrosarcoma cells (ATCC, Rockville, MD), C8161 melanoma cells and Eahy926 cells (derivative of HUVEC) were cultured in Dulbecco's modified Eagle's medium containing the antibiotics, 10 % fetal calf serum, and hypoxanthine/aminopterin/thymidine additive with the Eahy926 cells. Cultures of cells were harvested with trypsin-EDTA (endothelial cells) or EDTA alone (other cells), washed, and resuspended in the full serum-containing media as indicated above.

Random cell migration was studied using 8.0 µm pore size and 6.5 mm diameter Transwell inserts (Costar, Cambridge, MA) that were equilibrated in the serum-containing medium for 2 h before use. Tumor cell invasion was studied using

6.4 mm diameter Boyden chambers precoated with Matrigel (Becton Dickinson, Bedford, MA) and equilibrated in the serum-containing medium. 750  $\mu$ l of the serum containing media were added to the lower compartments of the migration apparatus. For random migration assays, cells were preincubated for 2 h in the presence of the peptides at the concentration indicated, and 20 000 cells in a volume of 100  $\mu$ l were plated in a Transwell. For Matrigel invasion, each well was plated with 100 000 cells in a 500  $\mu$ l volume with or without the peptides. After culturing cells for 16-20 h, cells were fixed in methanol, washed, and stained in toluidene blue. Cells were removed from the upper surface of the membrane with a cotton swab, and the cells migrated on the underside of the membrane were counted microscopically, or alternatively quantitated by scanning.

The CTHWGFTLC peptide was capable of blocking migration of a variety of cell lines studied in the presence of 10 or 20 % serum. In the Transwell random migration assay, the peptide inhibited concentration-dependently the motility of HT1080 fibrosarcoma cells (Fig. 5). At the concentrations of 500 and 100  $\mu$ g/ml, the peptide inhibited by 80 and 40 %, respectively. For the purpose of control, we synthesized a scrambled CWLTFTHGTC but could not use it because of its lack of solubility in aqueous buffers. We therefore used three unrelated highly soluble peptides EVGTGSCNLECVSTNPLSGTEQ, CQWNNDNPLFKEAEEVMPKFAES, and RAVRALWRC. None of these control peptides affected cell migration at a concentration of 500  $\mu$ g/ml (Fig. 5, and data not shown). CTHWGFTLC was not found to block cell surface integrins as the peptide did not prevent initial attachment and spreading of cells on fibronectin, collagen IV, or Matrigel substrata. No significant decrease in cell viability was noted after one or two-day culture of cells in the presence of the peptide (data not shown).

CTTHWGFTLC similarly inhibited random migration of C8161 melanoma cells, maximally by 80 % at the concentration of 500  $\mu\text{g/ml}$  (Fig. 6). The three control peptides did not affect cell migration.

5

We also studied the effect of CTTHWGFTLC on the random migration of endothelial cells in the Transwell assay (Fig. 7). At a concentration of 200  $\mu\text{g/ml}$ , the peptide showed 85 and 60 % inhibition of migration of Eahy 926 and HUVEC cells, respectively, and was still capable to partially inhibit at a concentration of 20  $\mu\text{g/ml}$ . The RAVRALWRC peptide did not cell block cell migration.

10

Finally, we examined the ability of CTTHWGFTLC to prevent Matrigel invasion of HT1080 and C8161 cells. In both cell lines, the peptide strongly suppressed invasion, and the inhibition was maximally 90 % at 500  $\mu\text{g/ml}$ , the highest concentration studied (Fig. 8). None of the three control peptides affected Matrigel invasion.

15

20

**Example 5. Suppression of growth of human breast carcinoma xenografts in athymic mice by locally applied CTTHWGFTLC**

Mice bearing human breast carcinomas were developed by inoculating  $1 \times 10^6$  MDA-B-435 cells in the fat mammary pad. After 4 weeks the volumes of the tumors were calculated by measuring the diameters in the three dimensions. The mice were divided in two groups each consisting of five animals. One group was treated with 200  $\mu\text{g}$  of CTTHWGFTLC in a 200  $\mu\text{l}$  volume administered three times a week adjacent to the tumor. The second group was given the cyclic peptide control CVRNSLAC. The tumor volumes were measured weekly; the results are after three-week treatment with the peptide (Fig. 9). The results show that CTTHWGFTLC peptide clearly inhibits breast carcinoma growth.

30

35

**Example 6.** Deactivation of proMMP-9 by CTTHWGFTLC as detected by gelatin zymography

5 C8161 melanoma cells were cultivated for 48 h in 24-well plates in medium containing 10 % serum. The CTTHWGFTLC peptide was included at the concentrations indicated in Fig. 10 (500 - 10  $\mu$ g/ml) and the control peptide RAV-RALWRC at 500  $\mu$ g/ml. The conditioned medium was analyzed by SDS gel electrophoresis followed by gelatin zymo-  
10 graphy. CTTHWGFTLC decreased concentration-dependently the levels of 82 kDa active MMP-9, but did not affect the levels of 72 kDa proMMP-2.

15 **Example 7.** Time-dependent induction of rounded cell morphology and detachment of the cells from the substratum

MB-435 breast carcinoma cells were cultivated for 48 h in 10 % serum-containing medium in the absence or presence of the CTTHWGFTLC peptide, after which cells were analyzed by light microscope. Unrelated synthetic peptides  
20 studied at the same concentrations had no effect on the morphology of cell layers. Rounded cell morphology is detectable within 16-24 h after applying CTTHWGFTLC but is not evident in short-time culture (Figs 11A to 11C);  
25 the peptide had no effect on the initial attachment of cells on the substratum during 1 or 2 h time scale.

**Example 8.** Effect of CTTHWGFTLC on cell viability

30 To assess the effect of CTTHWGFTLC on cell viability, 100 000 cells were plated in 24-well plates in 1 ml of medium containing 10 % fetal calf serum and 500  $\mu$ g/ml of CTTHWGFTLC or an unrelated control peptide. After culturing for 20 or 40 h, the viability was determined by  
35 staining with trypan blue, or with the MTT reagent according to the instructions of the manufacturer (Sigma, St. Louis). For cell adhesion studies, microtiter wells were

coated with fibronectin (Finnish Red Cross), type IV collagen (Sigma) or Matrigel, and blocked with BSA. Cells (100 000 cells per well) were incubated together with 500  $\mu$ g/ml of CTTHWGFTLC or a control peptide in a serum-free medium for 1 h. After washing twice with PBS, the bound cells were stained and counted.

The peptide was not found to affect cell number or viability by trypan blue dye exclusion, and has shown no toxicity when injected into animals. The peptide did not prevent initial attachment of cells on Matrigel, collagen or fibronectin.

**Example 9. Effect of CTTHWGFTLC on keratinocyte gelatinase production and expression**

30 000 HaCat cells (spontaneously transformed non-tumorigenic human keratinocyte cell line, Ryle et al., 1989) were seeded into the wells of 96-well plates (Nunc, Denmark) in 50  $\mu$ l of KGM and allowed to attach for 24 h in humidified atmosphere at 37°C. Then the cells were exposed to KGM or KGM containing 50-500  $\mu$ g/ml of CTTHWGFTLC with or without 10 ng/ml of TGF $\beta$ . A set of cultures were treated with 1, 10 or 20 ng/ml of TGF $\beta$  alone. After 24 h the medium was harvested and stored at -20°C until analyzed by zymography (Heussen & Dowdle, 1980). 12  $\mu$ l of the culture media were run in 10 % SDS-polyacrylamide gels containing 1.0 mg/ml 2-methoxy-2,4-diphenyl-3(2H)-furanone-labelled gelatin (O'Grady et al., 1984). The lysis of gelatin was monitored by long wave UV-light and the gels were photographed. A computerized densitometer (MCID-M4, Imaging research Inc., St. Catharines, Ontario, Canada) was used to measure the amount of gelatinases from the photographed gels. The cells in the plates were fixed with 4 % (v/v) formaldehyde in PBS containing 5 % (v/v) sucrose, and stained with 0.1 % crystal violet in boric acid (pH 6.0) for 20 min. After destain-

ing with 10 % acetic acid, the absorbancies were measured with Multiscan MS plate reader (Version 4.0, Labsystems, Helsinki, Finland) at 595 nm. The relative cell number obtained by this method was used when the amount of gelatinases per cell was counted. Only MMP-9 gave measurable cleavage rate in order to calculate the amount of the enzyme per cell. The results shown in Fig. 12 are mean of two experiments.

10 **Example 10.** Effect of CTTHWGFTLC on keratinocyte migration

24-well plates (Costar, Cambridge, MA, USA) were coated with 50 µg/ml of fibronectin (FN; from human plasma; Sigma, F-2006, St. Louis, MO, USA) in PBS (pH 7.4). Metal cylinders were placed into the coated wells and 50 000 HaCat cells in KGM media (in 50 µl) were seeded into the cylinders. The cells were allowed to attach to the substrate for 24 h at 37°C in humidified atmosphere. The cylinders were removed, and the non adherent cells were removed by washing with the culture medium, the medium was replaced with KGM containing various concentrations of CTTHWGFTLC or TGFβ. Cells were allowed to migrate out from the disk for 4 days at 37°C. The medium was harvested and cells were fixed with 4 % (v/v) formaldehyde in PBS containing 5 % (v/v) sucrose, and stained with 0.1 % crystal violet in boric acid (pH 6.0). The wells were photographed and the amount of migration was measured by counting the area of migrated cells using NIH Image 1.45 program for Macintosh computer. A photograph of the plates after 4 days of migration and calculated areas of migrated cells are shown in Fig. 13. The results are mean of two duplicate experiments.



**Sequence Listing Free Text**

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Variable aa, Xaa in position 9 can be any amino acid

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## SEQUENCE LISTING

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**Claims**

1. A matrix metalloproteinase inhibitor and down-regulator comprising the cyclic structure of the peptide motif

5

CXXHWGFXXC

wherein X is any amino acid residue.

10 2. The matrix metalloproteinase inhibitor and down-regulator according to claim 1 wherein the peptide motif is CRRHWGFEEFC.

15 3. The matrix metalloproteinase inhibitor and down-regulator according to claim 1 wherein the peptide motif is CTTHWGFTELC.

20 4. A pharmaceutical composition comprising a matrix metalloproteinase inhibitor and down-regulator according to any one of claims 1 to 3 and a pharmaceutically acceptable carrier.

25 5. The use of a matrix metalloproteinase inhibitor and down-regulator according to any one of claims 1 to 3 for the manufacture of a pharmaceutical composition for the treatment of matrix metalloproteinase (MMP) dependent conditions.

30 6. The use according to claim 5 for the manufacture of a pharmaceutical composition for the treatment of conditions dependent on MMP-2 and/or MMP-9.

35 7. A process for the preparation of a matrix metalloproteinase inhibitor/down-regulator according to claim 1, which process comprises solid-phase Merrifield peptide synthesis.

8. A method for the therapeutic or prophylactic treatment of matrix metalloproteinase dependent conditions in mammals

comprising administering to said mammal a matrix metallo-  
proteinase inhibitor/down-regulator according to any one of  
claims 1 to 3 in an amount which is effective in inhibiting  
and down-regulating MMP activations, expressions and/or  
5 functions in said mammal.

9. The method according to claim 8 for the therapeutic or  
prophylactic treatment of conditions dependent on MMP-2  
and/or MMP-9.

10

10. A method for inhibiting the formations, synthesis, ac-  
tivations, expressions, functions and actions of matrix me-  
talloproteinases in mammals, comprising administering to  
said mammal a matrix metalloproteinase inhibitor and down-  
15 regulator according to any one of claims 1 to 3 in an  
amount which is effective in blocking the formations, acti-  
vities, activations and actions of MMPs.

11. The method according to claim 10 for inhibiting the  
20 expressions, formations, activations and actions of MMP-2  
and/or MMP-9.

12. A method for inhibiting and down-regulating matrix me-  
talloproteinases *in vitro* comprising adding to an *in vitro*  
25 system a matrix metalloproteinase inhibitor and down-regu-  
lator according to any one of claims 1 to 3 in an amount  
which is effective in inhibiting and down-regulating the  
MMP activity.

13. The method according to claim 12 wherein the matrix  
30 metalloproteinases to be inhibited and down-regulated are  
MMP-2 and/or MMP-9.

14. The use of a matrix metalloproteinase inhibitor and  
35 down-regulator according to any one of claims 1 to 3 in  
biochemical isolation and purification procedures of matrix  
metalloproteinases.

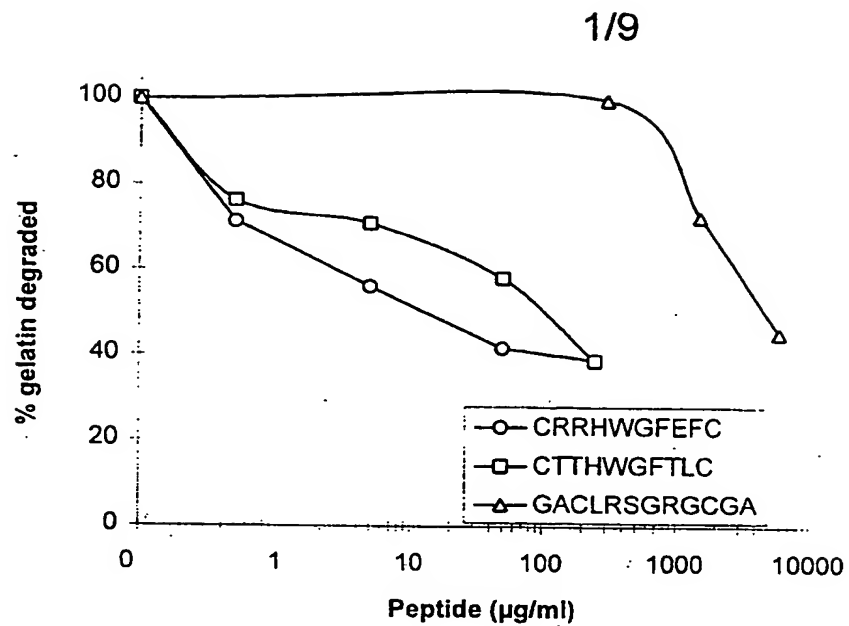


Fig. 1

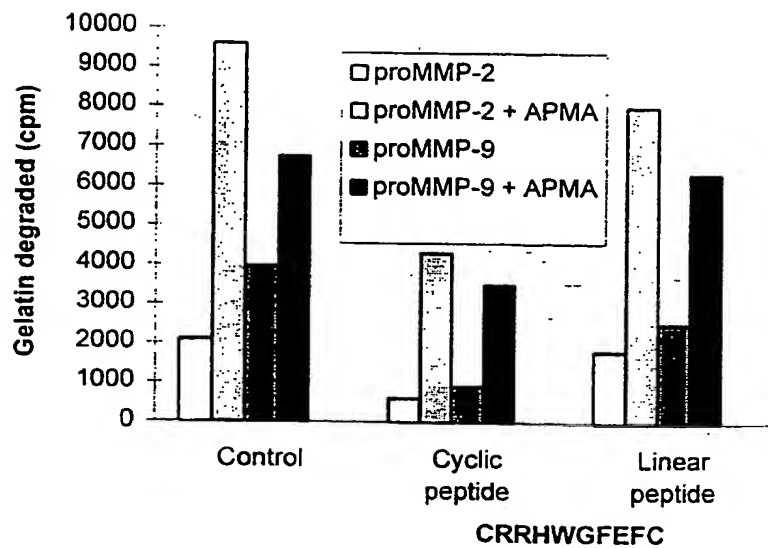


Fig. 2

2/9

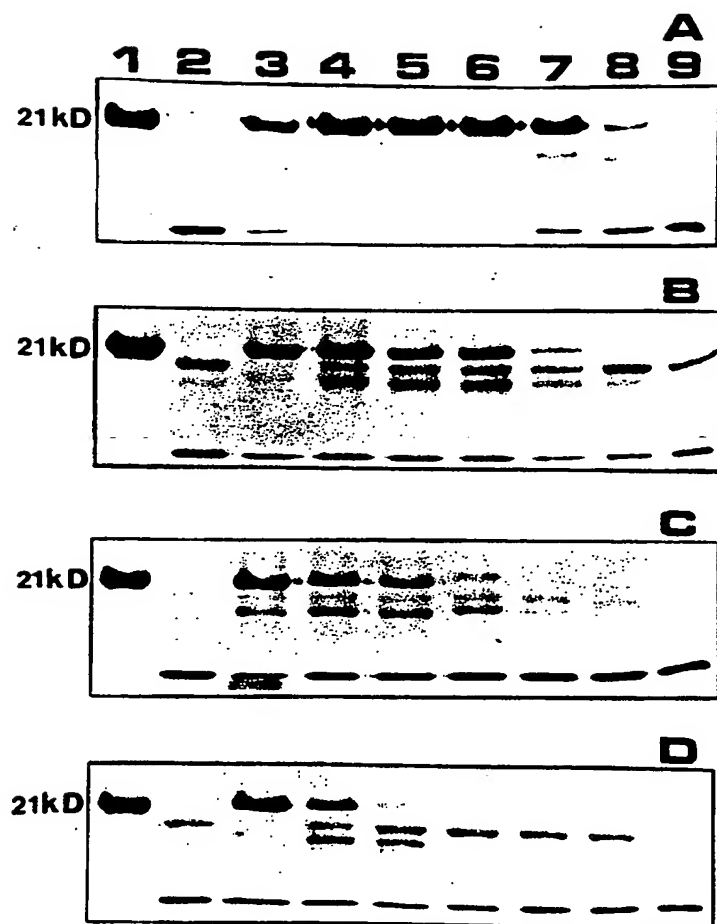


Fig. 3



3/9

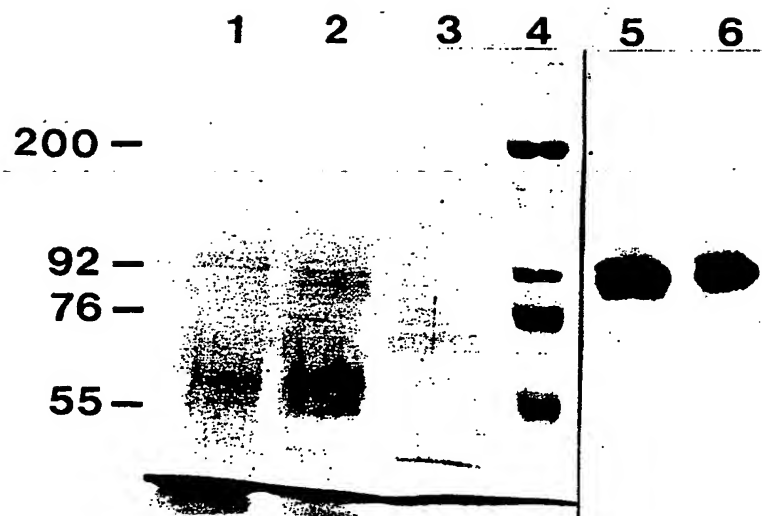


Fig. 4

4/9

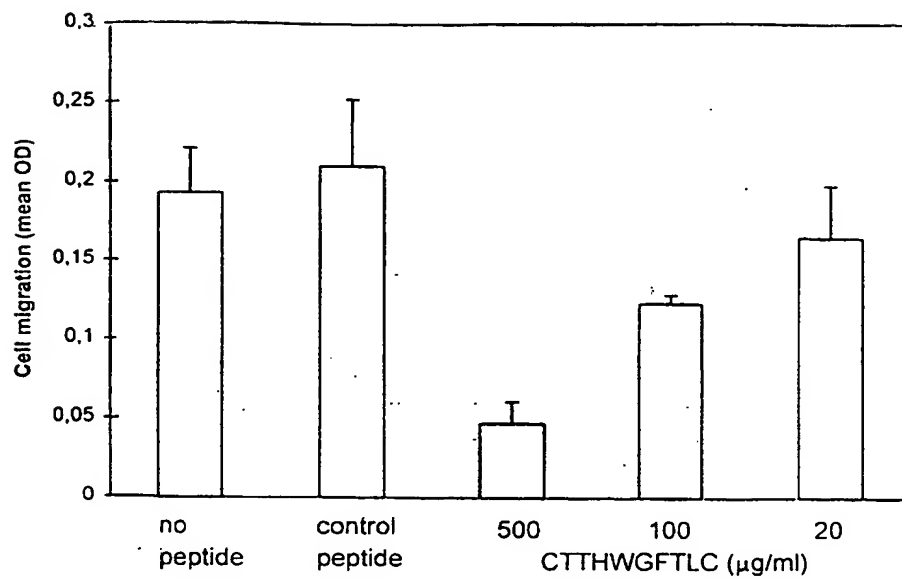


Fig. 5

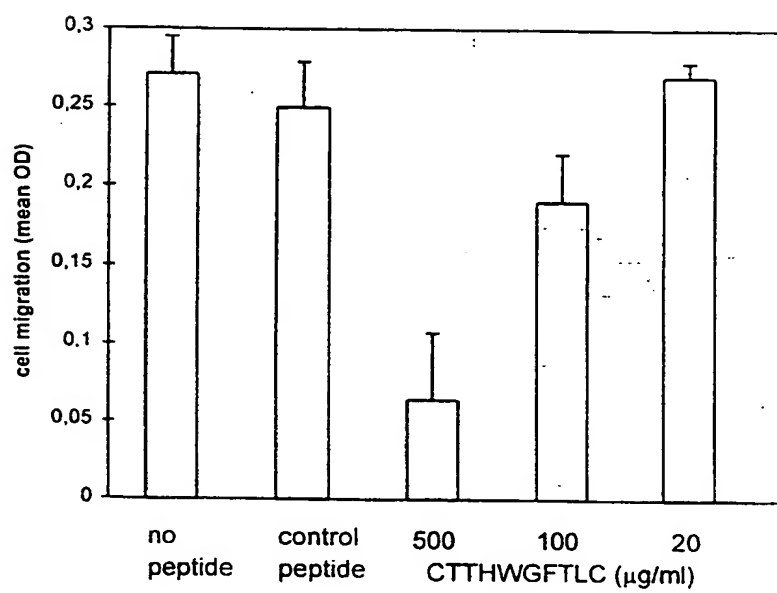


Fig. 6

5/9

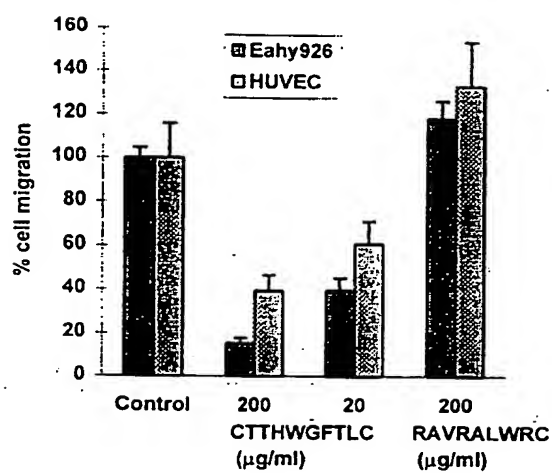


Fig. 7

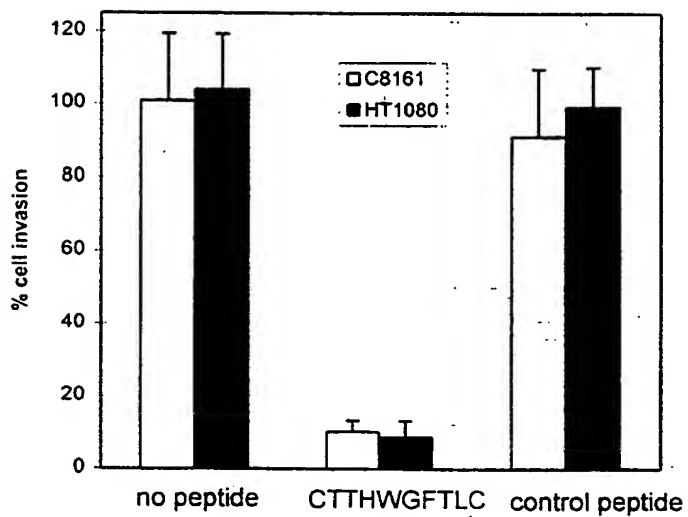


Fig. 8

6/9

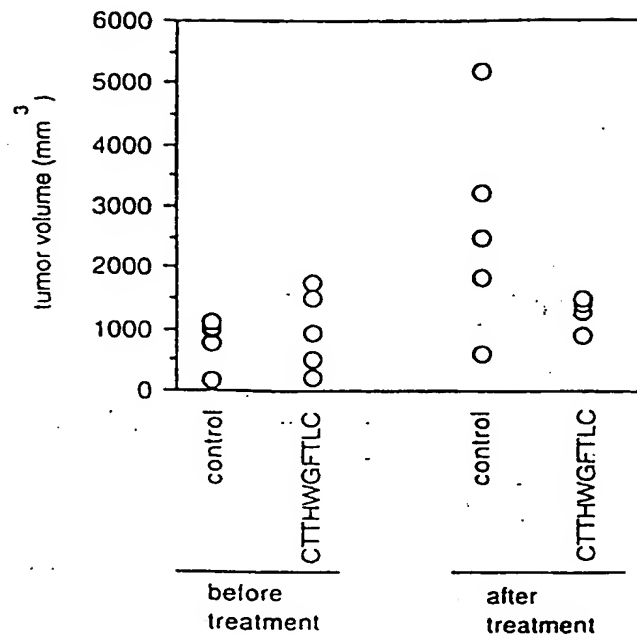


Fig. 9

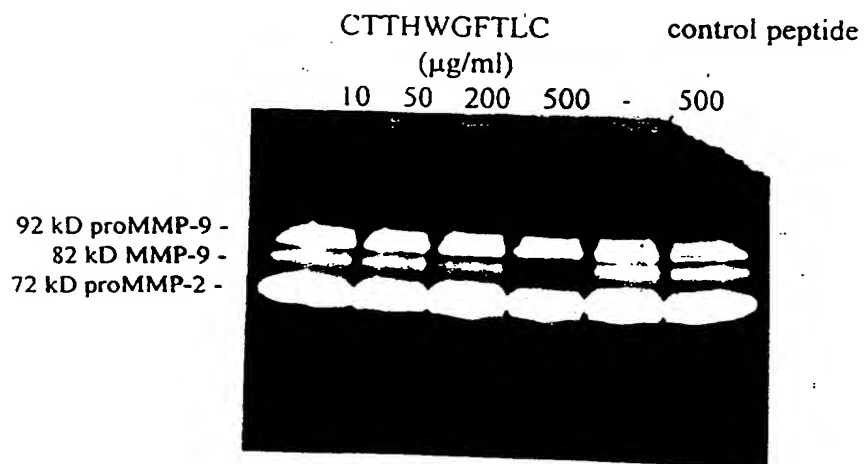


Fig. 10

7/9

MB-435 breast carcinoma cells grown for 2 days in 10 % serum

CTTHWGFTLC

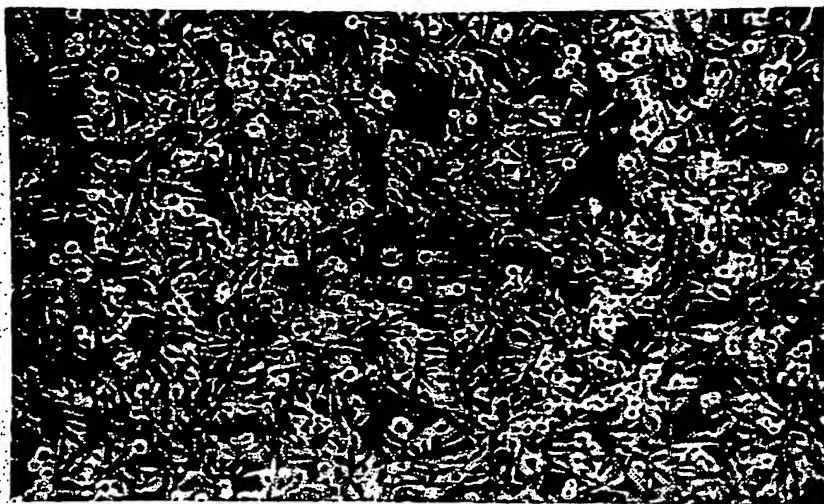
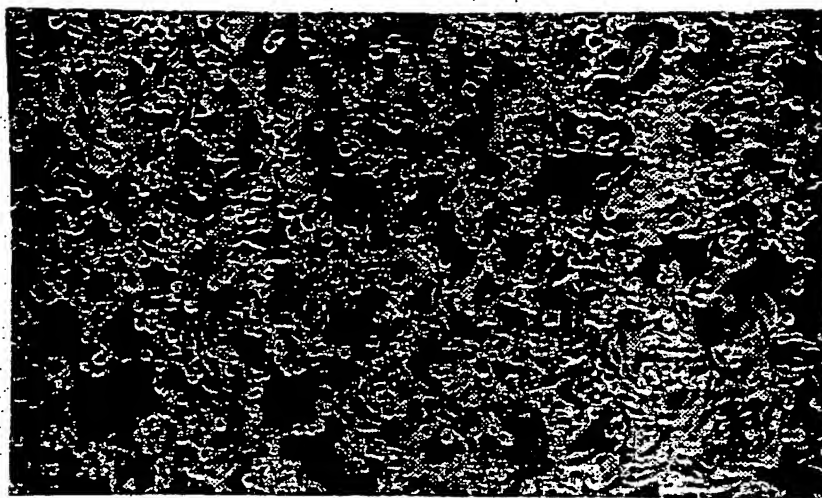
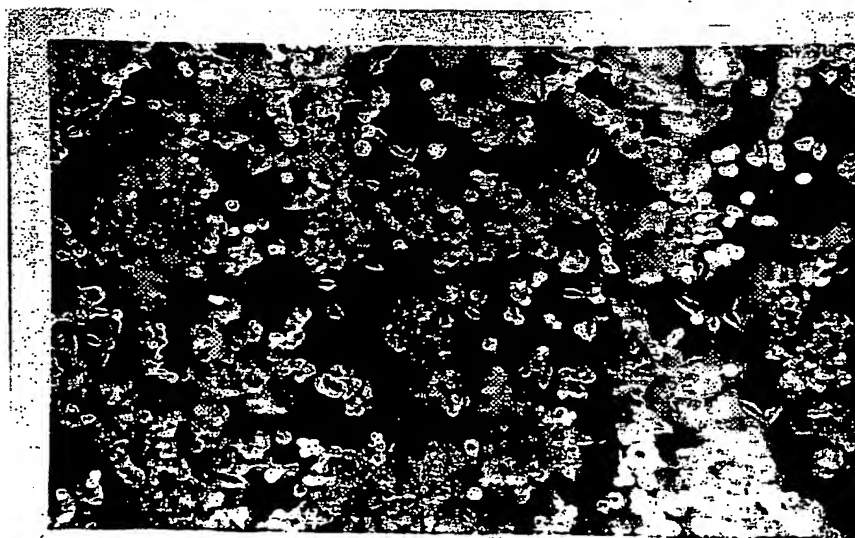


Fig. 11A



200 µg/ml

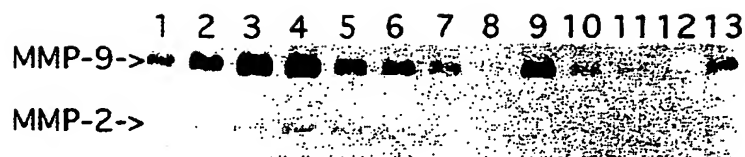
Fig. 11B



500 µg/ml

Fig. 11C

8/9

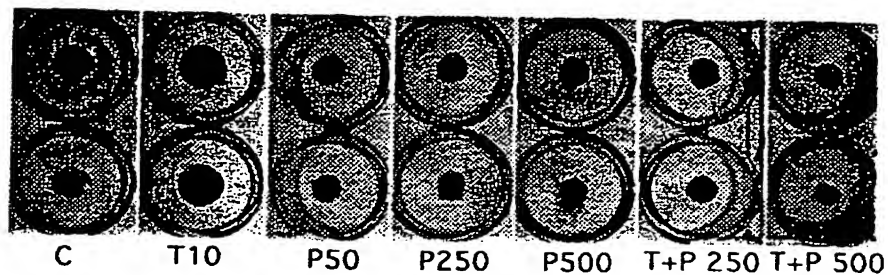


relative amount of MMP/  
relative cell number

1. Control	1.0
2. TGF $\beta$ 1 ng/ml	2.8
3. TGF $\beta$ 10 ng/ml	3.7
4. TGF $\beta$ 20 ng/ml	3.9
5. P291 50 $\mu$ g/ml	1.7
6. P291 100 $\mu$ g/ml	1.4
7. P291 250 $\mu$ g/ml	0.6
8. P291 500 $\mu$ g/ml	0.6
9. P291 50 $\mu$ g/ml and TGF $\beta$ 10 ng/ml	2.2
10. P291 100 $\mu$ g/ml and TGF $\beta$ 10 ng/ml	0.7
11. P291 250 $\mu$ g/ml and TGF $\beta$ 10 ng/ml	0.3
12. P291 500 $\mu$ g/ml and TGF $\beta$ 10 ng/ml	0.2

(P291 = CTTHWGFTLC)

Fig. 12



Calculated areas (5) of  
migrated cells

C	control cells	100 %
T10	TGF $\beta$ 10 ng/ml	139 %
P50	P291 50 $\mu$ g/ml	60 %
P250	P291 250 $\mu$ g/ml	69 %
P500	P291 500 $\mu$ g/ml	69 %
T + P250	TGF $\beta$ 10 ng/ml and P291 250 $\mu$ g/ml	76 %
T + P500	TGF $\beta$ 10 ng/ml and P291 500 $\mu$ g/ml	65 %

(P291 = CTTHWGFTLC)

Fig. 13

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 99/00204

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 7/06, A61K 38/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, REG, CAPLUS, MEDLINE, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9611209 A1 (CHIROSCIENCE LIMITED), 18 April 1996 (18.04.96)  -----	1-14

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"B" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date or another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

9 July 1999

Date of mailing of the international search report

12 -07- 1999

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI 99/00204

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 8-13  
because they relate to subject matter not required to be searched by this Authority, namely:  
**See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.**
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

01/06/99

International application No.

PCT/FI 99/00204

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9611209 A1	18/04/96	AT 179431 T	15/05/99
		AU 695796 B	20/08/98
		AU 3612795 A	02/05/96
		BR 9509237 A	21/10/97
		CN 1193978 A	23/09/98
		CZ 9700996 A	17/09/97
		EP 0784629 A,B	23/07/97
		FI 971412 A	04/04/97
		GB 9420057 D	00/00/00
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		NO 971537 A	04/06/97
		PL 319503 A	18/08/97
		ZA 9508396 A	07/10/96
		GB 9504907 D	00/00/00
		GB 9509431 D	00/00/00